



Characterization of loss-of-function and gain-of-function Eph receptor tyrosine kinase signaling in *C. elegans* axon targeting and cell migration

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Abstract

To understand how our brains function, it is necessary to know how neurons position themselves and target their axons and dendrites to their correct locations. Several evolutionarily conserved axon guidance molecules have been shown to help navigate axons to their correct target site. The *Caenorhabditis elegans* Eph receptor tyrosine kinase (RTK), VAB-1, has roles in early neuroblast and epidermal cell movements, but its roles in axon guidance are not well understood. Here, we report that mutations that disrupt the VAB-1 Eph receptor tyrosine kinase cause incompletely penetrant defects in axonal targeting and neuronal cell body positioning. The predominant axonal defect in *vab-1* mutant animals was an overextension axon phenotype. Interestingly, constitutively active VAB-1 tyrosine kinase signaling caused a lack of axon outgrowth or an early termination phenotype, opposite to the loss-of-function phenotype. The combination of loss-of-function and gain-of-function analyses suggests that the VAB-1 Eph RTK is required for targeting or limiting axons and neuronal cells to specific regions, perhaps by transducing a repellent or stop cue.

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Introduction

Our nervous system is the most complex of all the organ systems. This complexity arises from the billions of neurons that develop a highly organized pattern of connections, creating the neuronal network that makes up the functioning brain and the peripheral nervous system. How neurons position themselves and guide their axons and dendrites to their target sites is under intense research, and the molecular mechanisms are only starting to be elucidated. The process of axon guidance appears to be evolutionarily conserved throughout the animal kingdom (Araujo and Tear, 2003; Chisholm and Tessier-Lavigne, 1999), and a number of receptors and extracellular cues on the axons and surrounding substrates serve to promote or inhibit axonal outgrowth. Examples of these cues include Netrins, Slits, Ephrins, Semaphorins and Wnts, for reviews, see Dickson (2002) and Zou (2004).

The role of Ephrin and Semaphorin signaling in *C. elegans* axon guidance has not been well characterized. Instead, in *C. elegans*, the Semaphorins and their Plexin receptors function in epidermal enclosure and male tail sensory ray morphogenesis (Fujii et al., 2002; Ginzburg et al., 2002; Ikegami et al., 2004; Roy et al., 2000). Similarly, previous work on the single Eph receptor tyrosine kinase (RTK) VAB-1 and the 4 Ephrin ligands has focused on early embryonic cell movements, such as during neuroblast movements, enclosure of the ventral epidermis and male tail morphogenesis (Chin-Sang et al., 1999, 2002; George et al., 1998; Wang et al., 1999).

The study of vertebrate Eph receptor tyrosine kinases has provided significant insights into the mechanisms of axon guidance, cell adhesion and cell migration. For a recent review on Eph receptor signaling, see Pasquale (2005). The *C. elegans* VAB-1 Eph RTK and the ephrin ligands are expressed in the nervous system throughout development and in the adult. What role does VAB-1 play during axon guidance and postembryonic development? Previously, Zallen et al. (1999) examined the role of VAB-1 in axonal path finding and proposed that VAB-1 functions in parallel with the axon guidance receptor SAX-3/Robo. The Roundabout (Robo) family of receptors are

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evolutionary conserved and have been implicated in mediating axon guidance events, specifically axon repulsion upon binding its ligand Slit; for a review, see Wong et al. (2002). VAB-1 is also proposed to function with SAX-3 in a potential neuronal receptor complex to regulate embryonic morphogenesis and axon guidance (Ghenea et al., 2005). To extend the phenotypic analysis of Eph RTK signaling in axon guidance, we have characterized the neuronal and behavioral phenotypes of both loss-of-function and gain-of-function Eph receptor signaling in neurons.

Here, we report that *vab-1* mutants display two types of neuronal defects, an axonal defect where the predominant phenotype is an overextension of neurites beyond their target region and a defect in neuronal cell body positioning. We also constructed transgenic worms carrying a version of the VAB-1 tyrosine kinase that signals constitutively independent of ligand activity. These gain-of-function hyperactive VAB-1 animals have essentially 100% penetrant abnormal axonal phenotypes, and interestingly, the principal defects appear to be either growth failure or early axon termination phenotypes. Our results suggest that VAB-1 has roles in axon targeting and neuronal cell positioning, and that the VAB-1 RTK, like its vertebrate homologs, may transduce repulsive or stop signals during axonal outgrowth and targeting.

Materials and methods

Genetics and culture conditions

All *C. elegans* strains were manipulated as described by Brenner (1974). All alleles were isolated in the standard wild-type Bristol strain N2. All experiments were performed at 20°C unless otherwise indicated. Mutants used in the work are as follows: LGI: (*zdl5*)[*mec-4::gfp*] A GFP reporter expressed in the 6 mechanosensory neurons—kindly provided by Scott Clark (Clark and Chiu, 2003); LGII: *vab-1(e2, dx31)* (George et al., 1998), (*quls5*)[*mec-4::myr-vab-1*] (this work); LGIII: (*juls73*)[*unc-25::gfp*] (Hallam et al., 2000); LGIV: *efn-1(ju1)* (Chin-Sang et al., 1999), *efn-2(ev658)* (Wang et al., 1999), *efn-4(bx80)* (Chin-Sang et al., 2002), *unc-30(e318)* (Jin et al., 1994); LG V: *unc-34(e566)* (Brenner, 1974); LGX: *efn-3(ev696)* (Wang et al., 1999), *unc-115(mn481)* (Lundquist et al., 1998), (*kyls4*)[*ceh-23::gfp*] (Zallen et al., 1999), (*oxls12*)[*unc-47::gfp*] (McIntire et al., 1997); Not mapped or extrachromosomal array (this study): (*quls4*)[*mec-4::myr-vab-1(e2) G912E*]; (*quls8*)[*mec-4::myr-vab-1*]; (*quEx92*, *quEx95*, *quEx96*)[*mec-4::vab-1*]; (*quEx98*)[*unc-25::myr-vab-1*]; (*quEx107*)[*mec-4::myr-let-23*]; (*quEx108*)[*mec-4::myr-egl-15*]; (*quEx109*)[*mec-4::gfp-vab-1*]. Unless noted otherwise, all *C. elegans* strains were obtained from the *C. elegans* Genetics Stock Center, care of T. Stiernagle (U. of Minnesota).

Plasmid constructs and transgenic procedures

We incorporated the c-Src N-terminal myristoylation signal (MGSSKS) in VAB-1 using a PCR-based approach. A forward primer (oIC-101) encoding the myristoylation signal sequence and sequences to *vab-1* was used with a reverse primer to the 3' end of *vab-1* (oIC-96) to amplify a 1.6 kbp cDNA encoding the intracellular portion of VAB-1 (585 aa–1117 aa). The *mec-4* promoter (1.3 kbp) from pPD95.81 (Gift from Dr. Andy Fire's Lab) was subcloned into pPD96.41 (Dr. Andy Fire's Lab) replacing the *mec-7* promoter. The *myr-vab-1* PCR product was cloned behind the *mec-4* promoter to create *mec-4::myr-vab-1* (pIC-62). The *mec-4::myr-vab-1 G912E* (pIC-61) was constructed using a site directed mutagenesis three-step PCR approach. We designed complementary primers containing the missense mutation in *vab-1 G912E* and used these in two separate PCRs. The forward missense primer

(oIC-93) and a *vab-1* 3' primer (oIC-96) were used to amplify the *vab-1* cDNA 3' to the *G912E* mutation; and the reverse missense primer (oIC-94) and the 5' myristoylation primer (oIC-101) were used to amplify the *vab-1* cDNA 5' to the *G912E* mutation. These two PCRs were pooled and the 5' (oIC-101) and 3' (oIC-96) flanking primers were used to amplify a single *myr-vab-1* cDNA encoding the *G912E* mutation. This PCR product was cloned into the *mec-4* promoter vector as described for pIC-62. The *unc-25::myr-vab-1* construct (pICS-533) was made using a similar approach to the method for pIC-62, but instead, the *myr-vab-1* PCR product was cloned into the pSC35 (*unc-25* promoter vector at the *NheI/KpnI* sites, gift from Dr. Yishi Jin). The *mec-4::vab-1* (full length) construct (pIC-106) was made by amplifying the full-length *vab-1* cDNA (~3.3 kbp) product and sub-cloning it into pIC-62 replacing the *myr-vab-1* 1.6-kbp fragment behind the *mec-4* promoter. The *mec-4::myr-let-23* (pIC-269) and *mec-4::myr-egl-15* (pIC-267) constructs were created by using primers to amplify the cDNA regions encoding the intracellular domains of *let-23*(EGFR) and *egl-15* (FGFR) respectively and cloned into the *mec-4* promoter vector as described above. The *mec-4::gfp-vab-1* intracellular construct (pIC-347) was made by cloning a *gfp-vab-1* (~2.5 kbp) fragment into the *mec-4* promoter vector. All the PCR derived clones were verified by sequencing. Details of plasmid constructs and primer sequences are available upon request.

All *mec-4* promoter constructs were injected into *mec-4::gfp(zdl5)* animals at a concentration of 30 ng/μl, with the exception of the transgenic lines *quEx92* (pIC-106 construct injected at 5 ng/μl), *quEx95* and *quEx96* (pIC-106 construct injected at 15 ng/μl). Transgenic animals were identified by the co-injection marker pRF4/*rol-6* (30 ng/μl), or *odr-1::rfp* (30 ng/μl), and lines established as described by Mello et al. (1991). Extrachromosomal arrays carrying the various constructs were integrated onto chromosomes using UV/TMP ultraviolet and trimethylpsoralen method (Gengyo-Ando and Mitani, 2000). Two isolates of *mec-4::myr-vab-1 (quls5 and quls8)* were obtained and are phenotypically identical. One *mec-4::myr-vab-1(G912E) (quls4)* “kinase dead” integrant was used in this study.

Immunohistochemistry

Mixed stage animals were fixed and stained as described in Chin-Sang et al. (1999). Rabbit anti-VAB-1 antibodies (antigen VAB-1-HIS6) and mouse anti-phosphotyrosine 4G10 (Upstate Inc.) were used at 1:100 dilutions. FITC-conjugated goat anti-rabbit and Texas Red-conjugated goat anti-mouse or rabbit secondary antibodies (Jackson's Lab) were used at a 1:500 dilution.

Phenotypic analysis

To visualize the mechanosensory neurons in live animals, we used the *mec-4::gfp (zdl5)* reporter. In animals at the young adult stage, the PLML/R neuronal cell bodies are adjacent to each other and have axons that terminate in the middle of the animal near the vulva (Fig. 1). Worms were scored as having a PLM neuronal cell displacement if the cell was positioned anteriorly to the anal opening (Fig. 2A). Young adult animals were scored as displaying axon overextension defects if any of the PLM axons terminated at least six cell bodies (~81 μm) anterior to the vulva (Fig. 2C). Worms were scored as displaying premature termination defects if any of the PLM axons terminated at least three cell bodies (~40.5 μm) posterior to the vulva (Fig. 2C).

To visualize the GABA neurons (DDs and VDs), we used the *unc-25::gfp(juls73)* reporter strain (Jin et al., 1999). The plasmid (pICS-533) *unc-25::myr-vab-1* (30 ng/μl) was injected into the *unc-25::gfp* reporter strain along with pRF4 (30 ng/μl), and extrachromosomal lines were established.

To visualize the DVB neuron, we used the *unc-47::gfp (oxls12)* reporter in an *unc-30 (e318)* mutant background which eliminates GFP expression in the D type GABAergic neurons (Eastman et al., 1999; McIntire et al., 1997). Worms were scored as having an overextension defect if the DVB axon migrated dorsally away from the ventral cord once reaching the vulva.

To visualize the CAN neurons, we used the *ceh-23::gfp (kyls4)* reporter. Worms were scored as having posteriorly displaced neurons if either one of the CAN neurons traveled more than one CAN cell body (~12.6 μm) past the vulva.

The touch response was scored by gently brushing an eyebrow hair across the head or tail of the worm as described by Chalfie and Sulston (1981). Worms

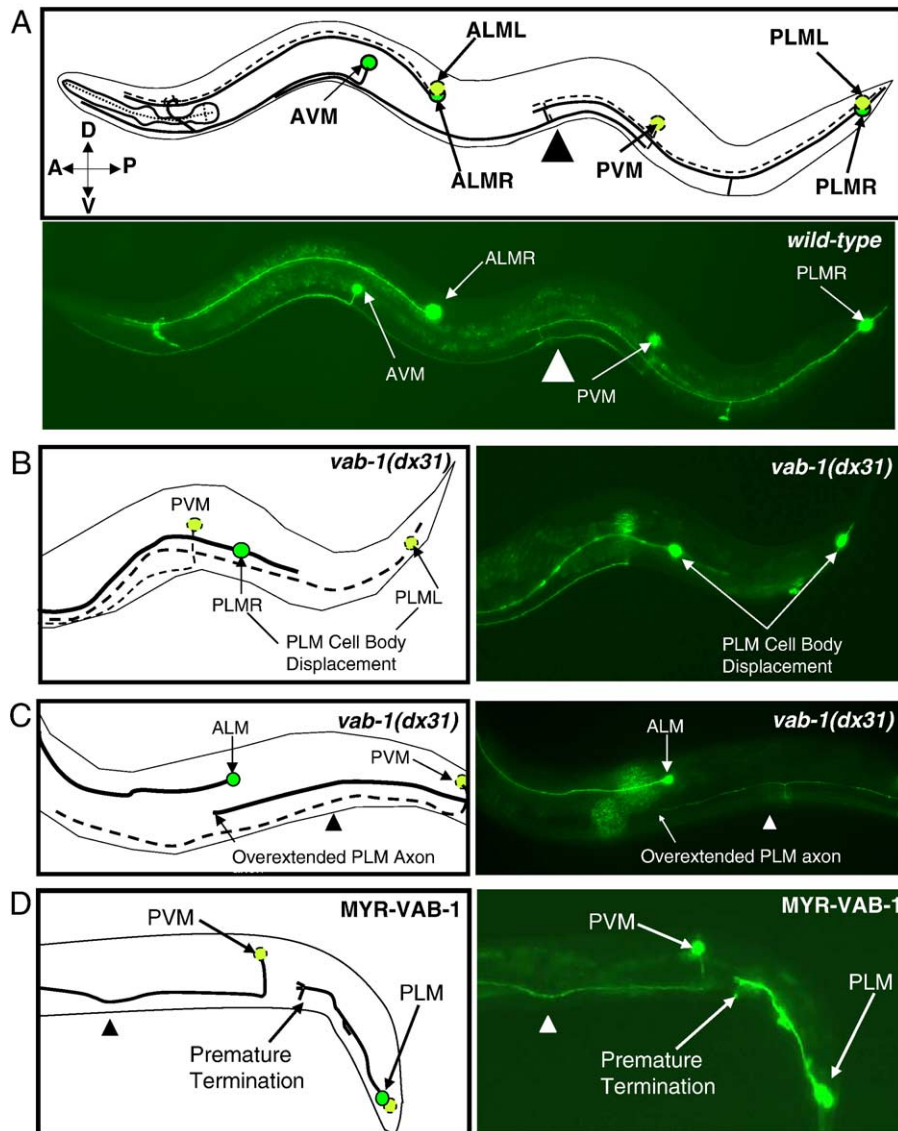


Fig. 1. Phenotype of mechanosensory neuron defects in *vab-1(dx31)* mutants and MYR-VAB-1 gain-of-function transgenic animals. All the panels show young adult animals. Line diagrams and GFP expression from the *mec-4* promoter are shown. (A) Wild-type cell body positions and neurite morphology of the 6 mechanosensory neurons. PLM (Right R and Left L) cell bodies are beside each other in the tail region. The PLM axons terminate around the vulva (triangle) region. Anterior/Posterior (A/P) and Dorsal/Ventral (D/V) are indicated. (B) *vab-1(dx31)* animals have PLM cell body position defects (PLMR is too far anterior). (C) *vab-1(dx31)* animals have PLM axons that overshoot past the vulva and ALM neurons. (D) Constitutively active MYR-VAB-1 caused a penetrant outgrowth or premature termination phenotype (opposite to the loss-of-function *vab-1* mutants). Note that PLM cell body positions are unaffected.

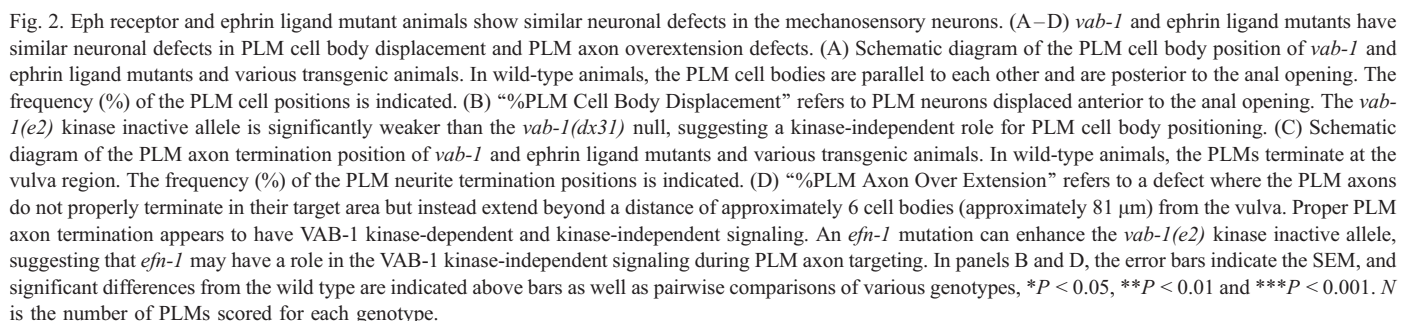
were scored as touch insensitive if the worms failed to move in the appropriate direction after two brushings.

Results

VAB-1 is required for proper neuronal cell positioning and axon targeting

To further investigate the role of *vab-1* in axon morphogenesis, we looked at three types of neurons: (1) mechanosensory neurons (Chalfie, 1993), (2) GABA (gamma-amino butyric acid) neurons (Jin et al., 1999) and (3) CAN (canal associated neuron) neurons (Sulston et al., 1983). These are well-characterized neurons in *C. elegans* with very stereotypical cell body positions and axon projections. We found that *vab-1* mutants have defects in both neuronal cell body

positioning and in axon targeting. To examine the mechanosensory neurons, we used a *mec-4::gfp* reporter (see Material and methods). The 6 mechanosensory neurons consist of a pair of PLMs (Right/Left) and PVM in the posterior and a pair of ALMs (Right/Left) and AVM in the anterior (Fig. 1A). All six neurons have well-defined cell body positions and neurite extensions. The paired PLM and ALM are located along the lateral body of the animal and send out axon processes that extend anteriorly. In wild-type adult animals, the PLM axons never extend past the ALM cell bodies and stop around the vulva area near the center of the animal (Figs. 1A, 2C). Both PVM and AVM are located at the lateral side of the animal and have axons that extend ventrally from their cell bodies to the ventral cord and then extend anteriorly (Chalfie and Sulston, 1981).



We analyzed two *vab-1* alleles. The *vab-1(dx31)* allele encodes a deletion of exons 1–4 and is therefore most likely a null allele, and *vab-1(e2)* encodes the point mutation G912E which appears to abolish the kinase activity (George et al., 1998; Wang et al., 1999). We observed two types of neuronal defects in *vab-1* Eph receptor mutants. The first defect was in neuronal cell body positioning. In wild-type animals, the PLM (Right/Left) neuronal cell bodies are positioned next to each other (Figs. 1A, 2A). In contrast, *vab-1(dx31)* null mutant animals displayed a PLM cell body displacement (36%), which at times reached a distance of up to 10 cell diameters from its normal position in the tail (Figs. 1B, 2A and B). The other defect was in axonal targeting. In *vab-1(dx31)* mutants 14% of the PLM axons extended too far anteriorly, stopping at or anterior to the ALM cell body (Figs. 1C, 2C and D). This axon overshooting suggests that VAB-1 may mediate a repulsive or stop signal in the PLM axons. Although we observed neuronal defects in the ALM neurons, we focused specifically on the PLMs (Right/Left), as these neurons are the easiest to score for overextension phenotypes. Since *vab-1* animals displayed defects in the mechanosensory neurons, we tested whether they could still respond to gentle touch. We found that *vab-1* animals still retained touch sensitivity, suggesting that the axon defects are not severe enough to disrupt connectivity of the mechanosensory neuron circuit. The *vab-1(e2)* mutation is significantly weaker than a *vab-1(dx31)* null allele when scored for embryonic lethality, suggesting that VAB-1 has kinase independent roles during embryogenesis (George et al., 1998). The PLM cell positioning defects in *vab-1(e2)* mutants occurred at a significantly lower frequency than that of the *vab-1(dx31)* null ($P < 0.001$) (Figs. 2A and B). *vab-1(e2)* mutants also exhibited overextended PLM axons at a lower frequency than the *vab-1(dx31)* null (Figs. 2C and D). Thus, as suggested for neuroblast movements during embryogenesis, VAB-1 may have both kinase-dependent and kinase-independent roles in the PLM axonal targeting and neuronal cell body positioning.

To determine if the expression of VAB-1 in the PLM is sufficient for proper axon targeting, we used the *mec-4* promoter to express full-length VAB-1 in the mechanosensory neurons of *vab-1(dx31)* null animals and ask whether the neuronal defects could be rescued. Three transgenic strains carrying a *mec-4::vab-1* transgene as a heritable, extrachromosomal array (*quEx92*, 95, 96) were isolated (see Materials and methods). The expression of VAB-1 in the mechanosensory neurons resulted in the partial rescue of the PLM axon overextension defects in *vab-1(dx31)* null mutants, however, VAB-1 expression in the touch neurons did not rescue the PLM cell displacement phenotype. Mutants carrying the *mec-4::vab-1* transgene exhibited overextension defects only at a frequency of 6–8% ($N = 500$) which was significantly less ($P < 0.05$) than the 14% overextension defects exhibited by the *vab-1(dx31)* null mutants. This suggests that *vab-1* can function in PLM axons to regulate their targeting.

We next examined whether *vab-1* is required for the proper neuronal cell positioning of neurons other than the mechanosensory neurons. We used a *ceh-23::gfp* reporter to observe the canal-associated neurons (CANs). The CAN neurons are

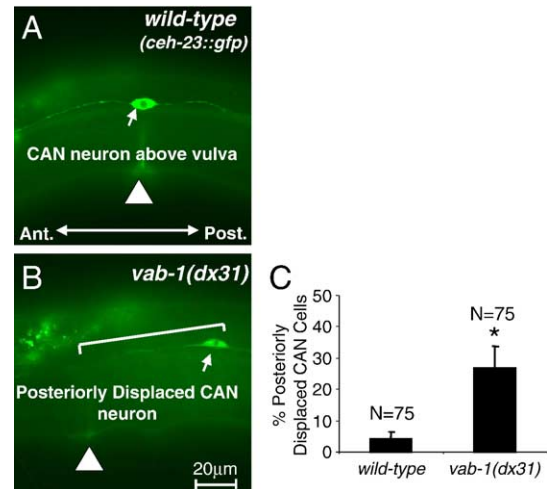


Fig. 3. *vab-1* mutant animals display CAN neuron positioning defects. Panels A and B show young adult wild-type and *vab-1* adult animals respectively. The CAN neurons were visualized by a *ceh-23::gfp* reporter. Anterior (Ant.) and Posterior (Post.) are indicated, and ventral is down. Arrowhead points to the vulva. (A) In wild-type animals during embryogenesis, the CAN neuronal cells migrate from the head region to a location just above the vulva region in the center of the animal. (B) *vab-1* mutants have posteriorly displaced CAN neuronal cells, demonstrating that the CAN neurons overshoot their target site. (C) “%Posteriorly Displaced CAN Cells” refers to CAN neuronal cells that have migrated past their target area by at least 1 cell body (~12.6 μm) from the vulva. Error bars indicate the SEM, and significant differences from the wild type were compared using the Student’s *t* test was * $P < 0.05$. *N* is the number of animals scored.

initially born in the head of the embryo and migrate posteriorly to the middle of the animal, approximately above the vulva and then extend neurites anteriorly and posteriorly (Fig. 3A). We observed a significant increase in the CAN neuronal cells migrating too far posteriorly (27%), overshooting their usual target area (Figs. 3B and C). *vab-1(dx31)* animals did not show any significant anterior displacement of the CAN neurons when compared to the strain carrying the *ceh-23::gfp* marker alone. The overshooting migration defect in the CAN neurons suggests that VAB-1 has a role in the positioning of the CAN neuronal cells and may be involved in transducing a repulsive signal, perhaps similar to the PLM axon termination signal.

To determine whether *vab-1* mutants exhibited overextension defects in other types of neurons, we analyzed the GABAergic DVB motor neuron. This neuron has an axon termination site in the middle of the animal similar to the PLMs. We used an *unc-47::gfp* reporter transgene in an *unc-30* mutant background to eliminate the D-neurons which allowed better visualization of the DVB neuron (Eastman et al., 1999). In wild-type animals, the DVB neuron is located in the dorso-rectal ganglion and extends an axon that migrates ventrally into the ventral cord. The DVB axon subsequently migrates anteriorly along the ventral cord and terminates at the vulva (Fig. 4A). In *vab-1(dx31)* animals, 13% ($N = 138$, Fig. 4B) of the DVB neuronal axons did not terminate at the vulva and migrated dorsally away from the vulva, therefore overshooting its usual area of termination. This dorsal extension was not observed in the *unc-30; unc-47::gfp* “wild-type” background ($N > 250$). The DVB axon overextension defect observed in *vab-1* mutants suggests that VAB-1 has a role in limiting the

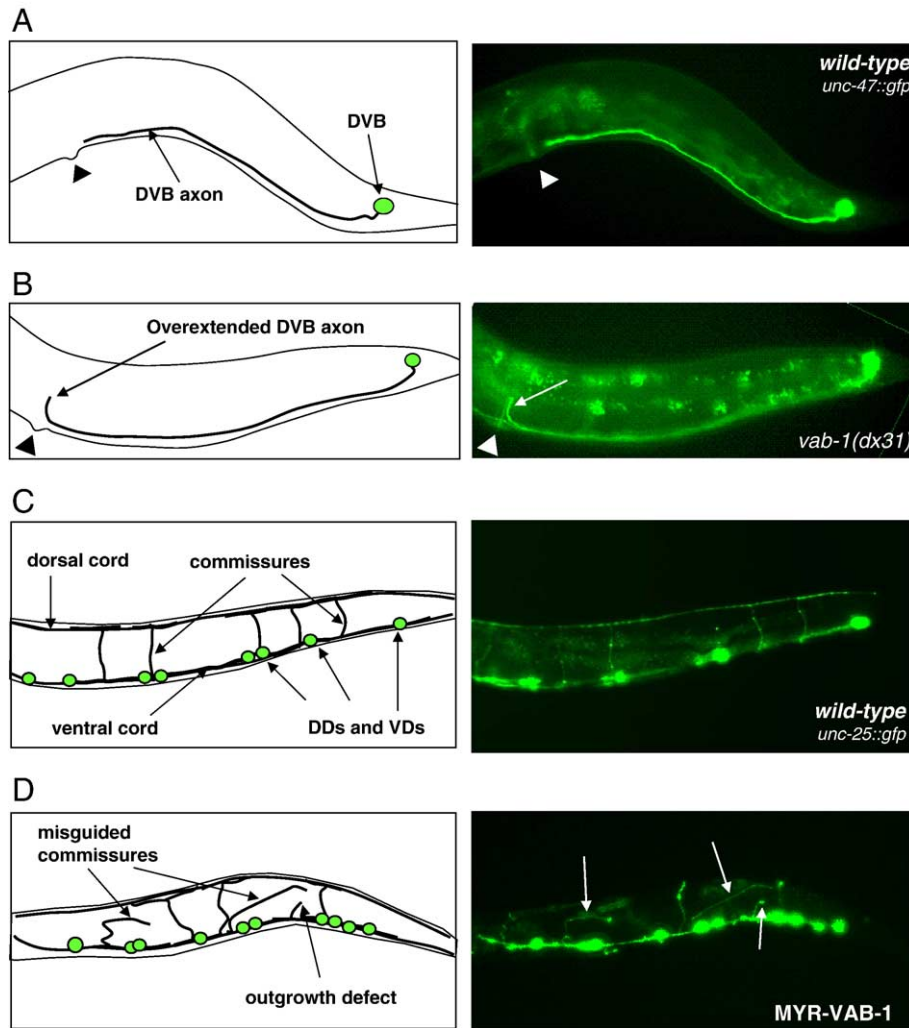


Fig. 4. VAB-1 loss-of-function and gain-of-function cause axon guidance defects in the GABA neurons. Panels show line drawings (left) and GFP expression (right) of the posterior region of young adult animals. Anterior is to the left, and dorsal is to the top. (A–B) The *unc-47* promoter was used to express GFP in *unc-30* animals to view the DVB GABA neuron. (A) Wild-type animals show a DVB neuronal cell in the dorso-rectal ganglion extending an axon into the ventral cord anteriorly and stopping near the vulva. (B) *vab-1* mutant animals show DVB axons that overextend beyond their termination region and migrate dorsally. (C–D) The *unc-25* promoter was used to express GFP and MYR-VAB-1 in the GABA neurons (VD and DD neurons). (C) Wild-type animals show DD and VD neuronal cell bodies on the ventral side that extend commissures from the ventral side to the dorsal cord. (D) Animals expressing MYR-VAB-1 in the GABA neurons show axon guidance defects in VD and DD neuron extension. Axons do not reach the dorsal cord (outgrowth defect) or wander (guidance defect).

DVB axon to a specific region, possibly in a similar fashion to the PLM axons. Interestingly, 5% of the *vab-1* mutants were missing the DVB or failed to express the GFP reporter in the DVB ($N = 278$), a phenotype that was never observed in the “wild-type” background.

Mutations in the ephrin ligands show a similar phenotype to that of vab-1 mutants

If VAB-1 is required for proper mechanosensory neuron cell body positioning and axon guidance, then mutations in its ligands, the ephrins (EFNs), might also show neuronal defects similar to those of *vab-1* mutants. The *C. elegans* genome contains four ephrins (EFN-1 through EFN-4) (Chin-Sang et al., 1999, 2002; Wang et al., 1999). All four ephrins have been shown to function with VAB-1 during embryogenesis, in addition, the fourth ephrin (EFN-4) is thought to have roles

independent of the VAB-1 Eph RTK (Chin-Sang et al., 2002), and EFN-2 but not EFN-1 is thought to be a ligand for VAB-1 during oocyte maturation (Miller et al., 2003). Previous work showed that the *efn-1* phenotype is much weaker than the *vab-1* null (Chin-Sang et al., 1999), and that *efn-2* and *efn-3* are even weaker than *efn-1* (Wang et al., 1999). Since the *efn-1* mutant phenotype is the strongest of the three ephrins and has embryonic phenotypes most similar to *vab-1* mutants, we first investigated whether the absence of EFN-1 resulted in neuronal defects. The *efn-1(ju1)* allele encodes a stop codon after 29 amino acids and therefore is likely to be a null allele (Chin-Sang et al., 1999). *efn-1(ju1)* mutant animals displayed neuronal defects in the mechanosensory neurons similar to *vab-1* animals. The *efn-1* animals exhibited defects in PLM neuronal cell body positioning, however, at a much lower (4%) frequency than those of both *vab-1(dx31)* and *vab-1(e2)* (Figs. 2A and B). The PLM displacement defects

of the *vab-1(e2);efn-1(ju1)* double mutant were not significantly different from the weaker *vab-1(e2)* allele alone (Figs. 2A and B). Thus, there seems to be at least a minor role for EFN-1 in PLM positioning. The *efn-1* mutants also exhibited PLM axon overextension defects at a frequency lower than the *vab-1(dx31)* null but similar to the weak *vab-1(e2)* allele. However, *vab-1(e2)* was significantly enhanced by *efn-1(ju1)* ($P < 0.001$) as the double mutant animals *vab-1(e2);efn-1(ju1)* exhibited PLM axonal overextension defects at a frequency similar to the *vab-1(dx31)* null (Figs. 2C and D). These results suggest that EFN-1 has a role in PLM targeting and may have a role in the kinase independent function of VAB-1 signaling during PLM axon targeting. We further investigated the effect of the absence of the remaining ephrin ligands by looking at an *efn-2(ev658) efn-4(bx80);efn-3(ev696)* triple mutant (Fig. 2). These three ephrin mutants are predicted to encode null proteins (Chin-Sang et al., 2002; Wang et al., 1999). The triple ephrin 2,3,4 mutant also showed similar neuronal defects as the *vab-1(dx31)* null. The triple mutant had PLM cell displacement defects at a lower frequency than the *vab-1(dx31)*, however, it showed higher PLM axon overextension defects when compared to *vab-1(dx31)* (Fig. 2), suggesting that these ephrins may have axon targeting roles independent of the VAB-1 receptor.

Gain-of-function constitutively active VAB-1 causes severe axon defects

To further characterize the axon guidance role of VAB-1 Eph RTK signaling, we created a gain-of-function constitutively active VAB-1 tyrosine kinase. Chimeric proteins that contain lipid-modifying signaling sequences can localize these proteins to the membranes and cause constitutively activated variants of such proteins (Reuther et al., 2000). We targeted the intracellular portion of VAB-1 (585 aa–1117 aa) to the plasma membrane by N-terminal myristoylation (MYR). This fusion protein is referred to as MYR-VAB-1 (see Materials and methods). We used the *mec-4* promoter to express MYR-VAB-1 in the mechanosensory neurons. Transgenic animals carrying *mec-4::myr-vab-1 (quls5)* exhibited dominant defects in the mechanosensory neurons that were more severe than those observed in the *vab-1* null. Homozygous *myr-vab-1 (quls5/quls5)* animals displayed defects that are essentially 100% penetrant (Fig. 5B), while heterozygous *(+/quls5)* animals have only 51% penetrance, suggesting that the *myr-vab-1* phenotype is sensitive to dosage of the *myr-vab-1(quls5)* transgene. The most obvious defect was an axon outgrowth failure or the premature termination of the PLM axons (Figs. 1D and 5A) (85.5%). Other axonal defects include axon guidance defects or wandering of the axon, often into the ventral nerve cord, and ectopic branching of axons. Note, unlike the *vab-1* null mutants, the MYR-VAB-1 did not significantly affect the PLM cell body positions (Fig. 2A). Since the *mec-4::myr-vab-1* transgenic animals displayed a completely penetrant axonal phenotype in the mechanosensory neurons, we asked whether these animals were abnormal in response to gentle touch. Indeed, *myr-vab-1 (quls5)* worms

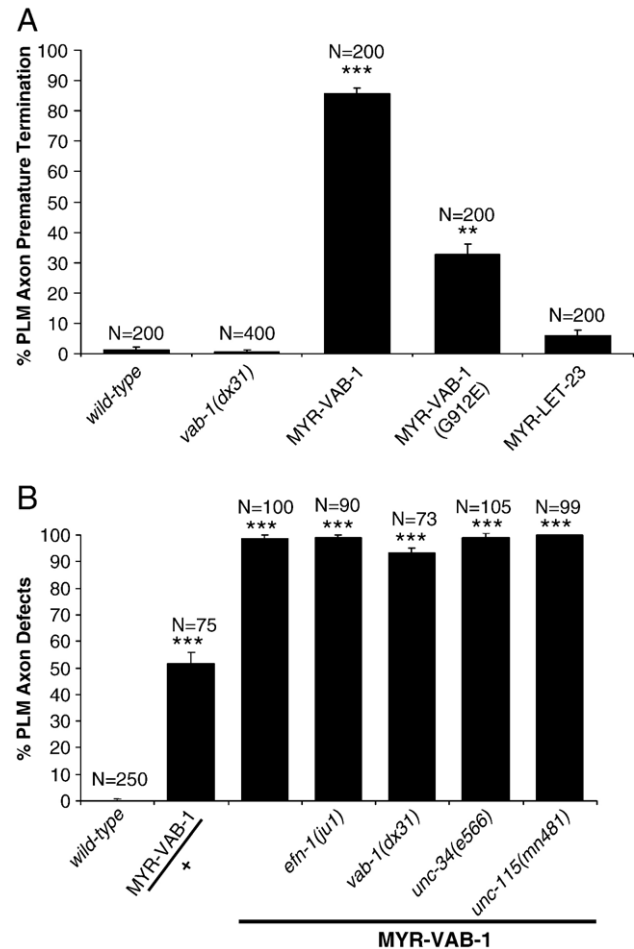


Fig. 5. A gain-of-function VAB-1 tyrosine kinase causes severe neuronal defects independent of endogenous EFN-1 and VAB-1 Eph RTK. (A) “%PLM Axon Premature Termination” refers to defects where the PLM axons do not extend to their target area but stop prematurely. Refer to Fig. 2C for termination positions. MYR-VAB-1 expressed in the mechanosensory neurons caused a strong early termination defect. In contrast, MYR-LET-23 (EGFR) did not cause significant premature termination, suggesting the defect is specific to VAB-1 tyrosine kinase. A point mutation in the VAB-1 kinase region (G912E) greatly reduces the neuronal defects, suggesting kinase activity is required for penetrant outgrowth defects. *N* is the number of PLMs scored for each genotype. (B) “%PLM Axon Defects” refers to the PLM axon premature termination and any other defects in axon migration such as wandering and branching. With the exception of “wild type”, all strains expressed MYR-VAB-1 and the indicated mutant alleles. The gain-of-function defects do not require a functional EFN-1 ligand or the endogenous VAB-1 Eph RTK. UNC-115 and UNC-34 are not required for VAB-1 gain-of-function signaling. Error bars indicate the SEM, and significant differences from the wild type were compared, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. *N* is the number of animals scored.

have altered behaviour as they show a touch insensitive phenotype (33% Anterior and 50% Posterior $N = 150$).

The defects caused by MYR-VAB-1 were shown to be independent of the endogenous VAB-1 Eph RTK and the EFN-1 ephrin ligand. Neither the *vab-1(dx31)* nor *efn-1(ju1)* null in a MYR-VAB-1 background were significantly different from the defects caused by MYR-VAB-1 (Fig. 5B). This further supports the idea that MYR-VAB-1 is a constitutively active VAB-1 molecule that does not require the ephrin ligand to modulate its activity nor is it regulated by endogenous VAB-1 activity.

To assess if *myr-vab-1* can affect axon development in other neurons, we used the *unc-25* promoter to target MYR-VAB-1 to the GABA neurons specifically the VD and DD motor neurons (Jin et al., 1999). MYR-VAB-1 (*quEx98*) expression resulted in axon guidance defects in the VD and DD neurons (Fig. 4D), indicating that the neuronal defects caused by MYR-VAB-1 are not specific to the mechanosensory neurons. The defects appear to be mainly outgrowth defects, reminiscent of the PLM early termination defects.

The tyrosine kinase activity is required for penetrant axonal defects in MYR-VAB-1

We used anti-VAB-1 and anti-phosphotyrosine antibodies to visualize MYR-VAB-1 and its activity. Transgenic animals carrying *mec-4::myr-vab-1 (quIs5)* specifically expressed MYR-VAB-1 in the touch neurons and appeared to be membrane localized when compared to a non MYR tagged version of VAB-1 (GFP-VAB-1 intra) (Figs. 6A and B).

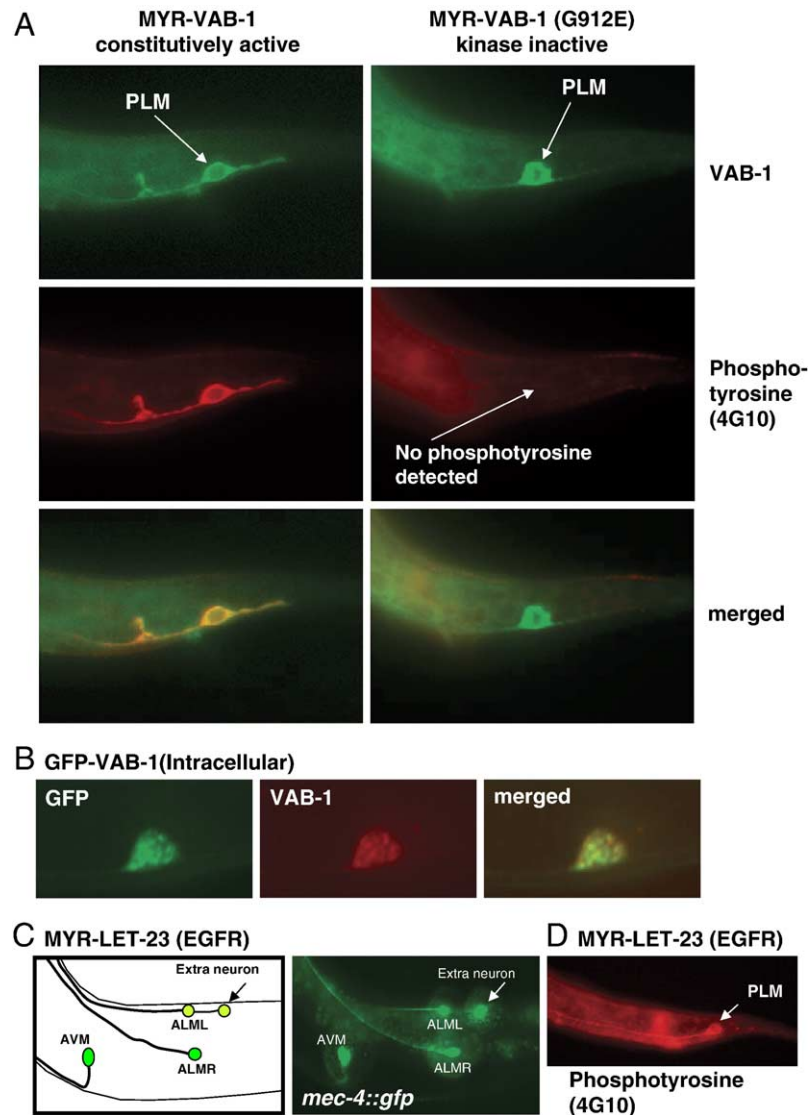


Fig. 6. Activated MYR-VAB-1 causes axon defects primarily through its kinase activity. (A, C–D) Wild-type MYR-VAB-1, a kinase inactive MYR-VAB-1 (G912E) version, and MYR-LET-23 were expressed under control of the *mec-4* promoter (mechanosensory neurons). (A) Wild-type MYR-VAB-1 (left) and MYR-VAB-1 (G912E) (right) are compared. Animals were co-stained for anti-VAB-1 (green) and anti-phosphotyrosine 4G10 (red). Animals that expressed the wild-type MYR-VAB-1 have outgrowth defects and express VAB-1 protein and phosphotyrosine in the mechanosensory neurons. The MYR-VAB-1 (G912E) kinase inactivating mutation still expressed MYR-VAB-1 to levels similar to that of wild-type versions, however, no phosphotyrosine is detected. Furthermore, the axonal termination defects are dramatically reduced, suggesting the VAB-1 kinase activity is required for the penetrant axonal defects observed. (B) Localization of a non-MYR tagged version of VAB-1. GFP-VAB-1 (intracellular region) was expressed in the mechanosensory neurons using the *mec-4* promoter and predicted to be cytoplasmic. A PLM neuron cell body is shown and the GFP-VAB-1 localized to cytoplasm as well as the nucleus. GFP fluorescence (green, left), anti-VAB-1 (red, middle) and merged (right) is shown. Furthermore, GFP-VAB-1 did not exhibit axonal termination defects. (C) MYR-LET-23 expressed in the mechanosensory neurons displayed defects not observed in MYR-VAB-1 animals. The *mec-4* promoter was used to express MYR-LET-23 in the mechanosensory neurons. Mechanosensory neurons were visualized by the *mec-4::gfp* reporter (right), and a line drawing is shown (left). ALMs appeared to be duplicated or extra neurons express the *mec-4::gfp* reporter. (D) MYR-LET-23 animals express similar levels of phosphotyrosine (anti-phosphotyrosine 4G10) in the touch neurons when compared to MYR-VAB-1, however, the PLM axon outgrowth defects are not observed, suggesting that defects seen in MYR-VAB-1 transgenic animals are specific to constitutive VAB-1 signaling.

Interestingly, the intracellular region of VAB-1 also appeared in the nucleus (Fig. 6B), but this was not observed in the MYR-VAB-1. To address whether the observed outgrowth and early termination defects were caused by a hyperactive VAB-1 tyrosine kinase, we created a mutant version of the MYR-VAB-1 that is predicted to abolish tyrosine kinase activity. We constructed a MYR-VAB-1 with a (G912E) missense mutation in the catalytic region of the VAB-1 tyrosine kinase. This is the same mutation encoded by the weak *vab-1(e2)* allele (George et al., 1998). Transgenic worms carrying a *mec-4::myr-vab-1 G912E (quIs4)* did not exhibit the penetrant neuronal defects observed in MYR-VAB-1 transgenic worms (Figs. 2C, 5A) (reducing the early termination phenotype from 85.5% to 32.7%). Anti-VAB-1 antibody staining of MYR-VAB-1 and MYR-VAB-1(G912E) transgenic animals revealed the presence of VAB-1 at a similar level of expression in the mechanosensory neurons (Fig. 6A). Therefore, the absence of penetrant neuronal outgrowth defects in animals carrying the MYR-VAB-1(G912E) version was not due to a lower expression level of VAB-1 in the mechanosensory neurons. Anti-phosphotyrosine antibody (4G10) confirmed the presence of phosphotyrosine in the mechanosensory neurons of MYR-VAB-1 transgenic animals but not in the mechanosensory neurons of MYR-VAB-1(G912E) transgenic animals (Fig. 6A). Therefore, we can conclude that the penetrant neuronal defects caused by MYR-VAB-1 are due mainly to hyperactive tyrosine kinase activity. To address the issue of whether the PLM termination defect is specific to VAB-1 Eph RTK signaling, we made analogous transgenic animals carrying either MYR-LET-23 (epidermal growth factor (EGF) RTK) or MYR-EGL-15 (fibroblast growth factor (FGF) RTK). Neither MYR-LET-23 nor MYR-EGL-15 animals showed the PLM axonal defects as seen in MYR-VAB-1 transgenic animals (Figs. 2A and C, 5A and data not shown). However, MYR-LET-23 did cause defects in the mechanosensory neurons not observed in MYR-VAB-1. For example, ALM morphology was abnormal, extending posterior neurites and had what appeared to be ALM duplications or ectopic expression of the *mec-4::gfp* reporter (Fig. 6C). Anti-phosphotyrosine antibody staining confirmed the presence of phosphotyrosine in the mechanosensory neurons in the MYR-LET-23 transgenic animals (Fig. 6D), suggesting that the PLM axon premature termination defects are not caused by just any constitutively active tyrosine kinase and are specific to VAB-1 tyrosine kinase signaling.

The VAB-1/Eph RTK may signal through a different pathway than UNC-40/DCC

Netrins are evolutionary conserved axon guidance molecules. In *C. elegans*, the UNC-40/DCC receptor is primarily used for signal attraction and outgrowth toward the UNC-6/Netrin cue. When MYR-UNC-40 is expressed in the mechanosensory neurons, it leads to a penetrant axon guidance phenotype including exuberant and excessive axonal outgrowth (Gitai et al., 2003). These axonal defects could be suppressed by loss-of-function mutations in *unc-115* (actin

binding protein) and *unc-34* (enabled/VASP actin regulator) (Gitai et al., 2003). To address whether these genes work in the VAB-1 pathway, we asked whether mutations in *unc-115* or *unc-34* could also suppress the MYR-VAB-1 defects. We crossed each mutant into our *mec-4::myr-vab-1(quIs5)* transgenic lines. However, neither *unc-115* nor *unc-34* mutants suppressed the MYR-VAB-1 axonal defects, suggesting that VAB-1 and UNC-40 receptors (at least in this context) function through a different downstream signaling mechanism (Fig. 5B). This also demonstrates that *unc-115* and *unc-34* are specific suppressors for ectopic UNC-40/DCC signaling and not just general suppressors of ectopic expression caused by the myristoylation anchor.

Discussion

The vertebrate Eph receptors and their ligands have diverse roles during development. The principal outcome of Eph-ephrin signaling appears to be a mutual repulsion during developmental programs such as hindbrain segmentation, neural crest cell migration and axon outgrowth and guidance, for review, see Pasquale (2005). To gain further insight on the role of Eph signaling in *C. elegans* axon guidance, we analyzed the effects of loss-of-function and gain-of-function VAB-1 Eph RTK signaling. Our results suggest that VAB-1 has roles in axon targeting and neuronal cell positioning and may act as a receptor for inhibitory or repellent signals during axonal outgrowth and migration.

Genetic redundancy in Eph RTK signaling during axon guidance

Complete loss of VAB-1 function causes only weak defects in axon guidance. This weak penetrance in axon guidance suggests that there is genetic redundancy in Eph RTK signaling during axon guidance. Work by Zallen et al. (1999) suggested that *vab-1* might act in parallel with *sax-3/Robo* and *unc-6/Netrin* in axon guidance. In addition, Hao et al. (2001) showed that *vab-1* and *slt-1* (ligand for SAX-3/Robo) double mutants have severe defects in head neuron axon guidance that are not present in the single mutations. During embryo morphogenesis SAX-3/Robo and VAB-1 are thought to function together as a putative receptor complex, and this complex may also occur in neurons during axon guidance (Ghenea et al., 2005). Two other proteins that are thought to act with VAB-1 during embryogenesis are EFN-4, a divergent ephrin, and PTP-3, a LAR receptor tyrosine phosphatase (Chin-Sang et al., 2002; Harrington et al., 2002). Double mutant combinations between *vab-1* and *efn-4*, *ptp-3* or *sax-3* lead to a synthetic lethal phenotype. It is possible that EFN-4 and PTP-3 and SAX-3 also function with VAB-1 during axon guidance, but because of the embryonic lethality in the double mutants, it precludes the observation of axon morphology later in development. Further work with partial loss-of-function alleles of these genes or mosaic or tissue specific knockouts will clarify whether these proteins function with the VAB-1 Eph RTK during axon guidance.

A role for VAB-1 in axon repulsion

In wild-type animals, the posterior PLM mechanosensory neurons extend neurites anteriorly but rarely extend past the ALM cell bodies and terminate around the vulva area at the center of the animal. Similarly, the DVB neuron extends its axon ventrally into the nerve cord and extends anteriorly to terminate at the vulva. Presumably, there must be receptors on the growth cone of the axon that sense and relay inhibitory or stop signals into the growing axon. Alternatively, there need not be a “stop cue” per se, and perhaps, there is some kind of intrinsic timing mechanism in PLM which tells the axon to stop growing. We have shown that *vab-1* mutants have weak defects in PLM and DVB axon morphology where the axon fails to terminate in its proper location and overshoots its target. Because VAB-1 encodes a receptor, this suggests that the VAB-1 Eph RTK may transduce an inhibitory signal that restricts axons to a specific region, and its disruption allows axons to overshoot that region. However, VAB-1 cannot be the only receptor that guides the PLM and DVB to their proper target site since the majority of the time these axons terminate at their proper location around the vulva. Other proteins known to be involved in PLM axon targeting include RPM-1, a putative guanine nucleotide exchange factor with a RING H2 finger (Schaefer et al., 2000); SAX-1, an NDR cell morphology-regulating kinase; and SAX-2, a large conserved protein with HEAT/Armadillo repeats that functions with SAX-1 (Gallegos and Bargmann, 2004). We have also observed PLM overextension defects in SAX-3/Robo mutants (unpublished). Our observation of PLM axon targeting defects in *vab-1* and *sax-3* mutants is analogous to HSN midline crossing observed by Zallen et al. (1999) and suggests that VAB-1 and SAX-3 may contribute to common axon guidance signaling pathways that are used for axon repulsion.

Cell movements and axon guidance share similar mechanisms

VAB-1 and EFN-1 have established roles in cell migrations during embryogenesis (Chin-Sang et al., 1999; George et al., 1998). Since the PLM neurons in wild-type animals are normally in their correct locations at hatching, this implies that the cell body displacement of the PLM in *vab-1* animals occurs during embryogenesis. This is consistent with the previously reported role of VAB-1 in early neuroblast movements. Perhaps the cell body displacement of PLM reflects defects in the early embryonic cell movements. So if the precursor neuroblasts of PLM are initially mispositioned, so are its daughter cells. Thus, in this sense, VAB-1 may not act cell autonomously in the PLM for cell positioning. This may also explain why *mec-4::myr-vab-1* animals did not display PLM cell displacement defects because the *mec-4* promoter is expressed too late after the PLMs are born. This is also consistent with why the *mec-4::vab-1* transgene did not rescue the PLM cell body position defects but had rescuing activity in the PLM axonal targeting. The Ephrin mutant, *efn-1(ju1)*, also has similar defects to that of *vab-1* null mutants and could significantly enhance the weak *vab-1(e2)* mutant in the PLM

overextension phenotype. This suggests that EFN-1 may play a role in VAB-1 kinase-independent signaling during PLM targeting. It is also possible that the PLM axonal defects arise from defects only in “forward” or kinase-dependent signaling, and perhaps, the *vab-1(e2)* mutant retains some residual kinase activity. In this case, the *vab-1(e2)* with weak kinase activity would still be activated by ephrin ligands, however, in the absence of EFN-1, the “forward” signaling would be reduced or not occur at all, resulting in an enhanced phenotype. *efn-1(ju1)* animals also have PLM cell body displacements but at a lower frequency than *vab-1* null animals. Interestingly, *efn-1(ju1)* did not enhance the *vab-1(e2)* mutants in the PLM cell movements, which suggests that the other ephrins or other ligands may be involved in this process. Indeed the frequency of the PLM cell positioning defects and PLM axon overextension defects observed in the *enf-2 efn-4;efn-3* triple mutant indicates that the remaining ephrins are required for proper neuronal development of the PLM neuronal cells and axons.

Unlike the PLM neurons, the CAN neuronal cells do not remain in their location after they are born in the embryo but undergo a long distance migration to the middle of the animal. Hence, the migration defects of the CAN neuronal cells in *vab-1* mutants may be due to the absence of a VAB-1 signal to prevent CAN cells from migrating into the restrictive region. Interestingly, *sax-3/Robo* mutants exhibit epidermal and neuroblast migration defects similar to *vab-1* mutants, but the CAN neuron cells are anteriorly mispositioned, which is opposite to the posterior mispositioning defects observed in *vab-1* mutant animals (Ghenea et al., 2005; Hao et al., 2001). Nevertheless, it suggests that both the VAB-1/Eph RTK and the SAX-3/Robo receptors have roles in CAN neuronal cell positioning.

The ability of a guidance cue to regulate both cell migration and axon guidance is a common strategy used during development of the nervous system (Hinck, 2004). For example, the Slit ligands and their Robo receptors are best known for their roles in axon guidance, specifically axon repulsion at the midline. However, Slit also has roles in cell migration similar to what is proposed for Eph RTK signaling in rat anterior subventricle zone (SVZ) neuronal cell migrations (Wu et al., 1999) and chick neural crest cells (Jia et al., 2005). Here, Slit acts as a repulsive cue for migrating neurons which is similar to how ephrins prevent the migration of neural crest cells on caudal somites (Krull et al., 1997; Smith et al., 1997; Wang and Anderson, 1997). The ability of Slit to play a repulsive role in early cell migrations and in axon guidance suggests the presence of a common molecular guidance mechanism between neuronal cell migrations and axonal guidance. Similarly, VAB-1 Eph RTK may use common guidance mechanisms in organizing the early neuroblast and epidermal cell movements during embryogenesis and then later act as a receptor during axon guidance.

Targeting the Eph RTK to the cell membrane causes constitutive kinase activity

We suggest that targeting the VAB-1 intracellular domain to the inner membrane by N-terminal myristoylation caused a

constitutively active tyrosine kinase. A similar approach was used to anchor the Met receptor tyrosine kinase (Kamikura et al., 2000). However, in that report, Kamikura et al. used the myristoylation signal on a tyrosine kinase that was already constitutively active (Tpr-Met oncoprotein), but nonetheless, they showed that membrane localization of the Tpr-Met oncoprotein enhanced cellular transformation (Kamikura et al., 2000).

The premature axon termination defect caused by MYR-VAB-1 indicates that an overactive VAB-1 kinase may result in growth cone collapse or retraction, a phenotype that is opposite to the *vab-1* loss-of-function mutants. The premature termination axon phenotype is consistent with the known roles of vertebrate Eph RTKs in promoting axonal repulsion. For example, ectopic ephrin A5 can cause growth cone freezing and axonal back-branching (Davenport et al., 1999). Similarly, in addition to the premature termination defect seen in axons expressing MYR-VAB-1, we see many axons that sprout ectopic branches. This branching phenotype may represent a consequence of the outgrowth phenotype, where the axon stalls or terminates and so starts a new branch. Recently, Depaepe et al. showed that overexpression of EphA receptor function caused neural progenitor cell apoptosis, resulting in premature depletion of progenitors and a subsequent dramatic decrease in brain size (Depaepe et al., 2005). However, we did not see any evidence of apoptosis in the mechanosensory neurons when MYR-VAB-1 was expressed from the *mec-4* promoter. This might be expected as ephrin/Eph RTKs have diverse roles in development and may control very different functions depending on their cellular context. Interestingly, we did notice that in *vab-1* null mutants, the DVB neuron was absent in 5% of the animals, which could possibly be due to apoptosis, a cell fate change or a failure of the GFP reporter to express in DVB. Further investigation should clarify these possibilities.

Eph RTKs including VAB-1 have been implicated in reverse signaling through their extracellular domains. However, since the MYR-VAB-1 protein does not contain any extracellular domains, we suggest that all the defects are due to the “forward signaling” of VAB-1. When we express MYR-VAB-1 exclusively in the mechanosensory neurons, we detected tyrosine kinase activity in the mechanosensory neurons, which is not normally seen in the mechanosensory neurons of wild-type worms. Furthermore, we did not see any tyrosine kinase activity when the entire VAB-1 receptor was expressed in the mechanosensory neurons. This suggests that the membrane targeting via the myristoylation signal most likely induces a constitutive VAB-1 tyrosine kinase through a ligand-independent dimerization. In contrast, the full-length VAB-1 Eph RTK may still require an active ligand for activation. Expressing MYR-LET-23 (EGFR) or MYR-EGL-15 (FGFR) in the mechanosensory neurons did not result in PLM axonal targeting defects, suggesting that the defects seen in MYR-VAB-1 transgenic animals are specific to VAB-1 constitutive signaling. Interestingly, MYR-LET-23 (EGFR) caused defects in the touch neurons not seen in MYR-VAB-1. For example, ALMs appeared to be duplicated or extra cells would express the *mec-4::gfp* reporter. This also provides support for the

specificity of each hyperactive tyrosine kinase as it demonstrates that its downstream signaling events lead to different phenotypes.

The MYR-VAB-1 (G912E) version of the protein did not display any kinase activity, and although it greatly reduced the early termination defects observed in the wild-type MYR-VAB-1, it did not suppress the defects completely. A simple explanation for the outgrowth defects in the MYR-VAB-1(G912E) version is that the other domains of the intracellular region of VAB-1, such as the PDZ (PSD95-Disc large-Zonula occludens) and a weak SAM (Sterile Alpha Motif) binding motif, are still able to function in the VAB-1 forward signaling pathway in a manner that is independent of the kinase domain. This could also explain why the *vab-1(e2)* allele is a weak allele, even though it is predicted to have an inactive kinase domain. Alternatively, it is possible that the G912E mutation does not completely abolish the kinase activity, and there is some weak tyrosine kinase signaling in these mutants that is beyond the limits of our detection.

A genetic tool to isolate effectors of VAB-1 signaling

Since *vab-1* null animals display incompletely penetrant phenotypes, it makes it very difficult to carry out classical suppressor analysis on *vab-1* mutants. We show that an overactive Eph tyrosine kinase leads to stronger defects in the nervous system when compared to a null mutation. Similarly, constitutively active mutations in the *C. elegans* Rac-like proteins MIG-2 and CED-10 resulted in more severe defects in migration and axon path finding than the loss-of-function mutations (Lundquist et al., 2001; Zipkin et al., 1997). Constitutively activated RTKs may cause dominant phenotypes by an overstimulation of the signal transduction pathway. We have shown that animals that carry a hyperactive MYR-VAB-1 in the mechanosensory neurons display neuronal defects with complete penetrance, and a point mutation that affects the kinase activity can dramatically suppress this phenotype. Therefore, we can use these transgenic animals as a starting strain for the isolation of suppressors. Such suppressors are likely to encode potential downstream effectors of VAB-1 signaling. This type of suppressor of ectopic gene activity has been used successfully in *C. elegans* for the UNC-5 and UNC-40 Netrin receptors (Colavita and Culotti, 1998; Colavita et al., 1998; Gitai et al., 2003).

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